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ITALIAN CHEESE RIPENING. VI. EFFECTS OF DIFFERENT TYPES OF LIPOLYTIC ENZYME PREPARATIONS ON THE ACCUMULATION OF VARIOUS FREE FATTY AND FREE AMINO ACIDS AND THE DEVELOPMENT OF FLAVOR IN PROVOLONE AND ROMANO CHEESE[1,2]

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Previous papers in this series have dealt with the concentrations of various free amino and fatty acids in commercial Provolone and Romano cheese *(3, 4)*. Fat degradation, specifically the production of free butyric acid, appeared to be directly related to the type of enzyme product used in the manufacture of cheese *(2, 3, 4)*. In contrast, the free amino acid content was not related to the enzyme products used but to the action of the bacterial flora *(3)*.

However, the significance of these findings could not be evaluated, since the cheeses were made in various factories under different conditions. Therefore, this study was made in order to evaluate the effects of different types of lipolytic enzyme products on the ripening of Italian cheese made in one factory under controlled conditions.

PROCEDURE

Four vats of Romano and four vats of Provolone cheese were manufactured from the same milk supply and the same starter culture *(Lactobacillus bulgaricus)*. The only variable introduced was the type of enzyme preparation used for each of the four cheeses. For both Romano and Provolone, one lot each was made with the following: *(a)* rennet extract alone (Hansen), *(b)* rennet extract (Hansen) plus calf glandular preparation (Italase), *(c)* rennet extract plus kid glandular preparation (Capalase), and *(d)* imported crude kid rennet paste.

Each variety of cheese was manufactured by a commercially recognized method and in a commercial cheese factory. The manufacturing methods for each lot of each specific variety were maintained constant, so that the temperatures and vat acidities were identical for all lots at any specific stage of the manufacturing process. The manufacturing details are shown in Tables 1 and 2 for Provolone and Romano, respectively. The Provolone cheese was smoked and

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TABLE 1

Manufacturing data for a typical Provolone cheese

Amount of milk	200 lb.
Fat test of milk	3.3%
Acidity of milk	0.19%
Acidity of starter	1.3%
Amount of starter	1.5% *(Lactobacillus bulgaricus)*
Amount of rennet	3 oz/1,000 lb. of milk
Amount of lipase	4 oz/1,000 lb. of milk
Temperature at setting	90° F.
Time to set	27 min.
Total time to cook	30 min.
Temperature of cooking	117° F.
Time from setting to draining	65 min.
Acidity at draining	0.16%
Time from setting to mixing	150 min.
Temperature of mixing water	165° F.
Temperature and time of mixing curd	Average of 140° F. for 25 min.
pH at mixing	5.3
Salting	Brine salted

TABLE 2

Manufacturing data for a typical Romano cheese

Amount of milk	200 lb.
Fat test of milk	2.5%
Acidity of milk	0.185%
Acidity of starter	1.3%
Amount of starter	1.5% *(Lactobacillus bulgaricus)*
Amount of rennet	3 oz/1,000 lb. of milk
Amount of lipase	4 oz/1,000 lb. of milk
Time from setting to cut	25 min.
Temperature of cooking	115° F.
Total time to cook	30 min.
Time from setting to draining (1st drain)	60 min.
Time from setting to draining (2nd drain)	90 min.
Time from setting to dipping curd	100 min.
pH at dipping	6.1 (0.23% acidity)
pH out of forms	5.3
Salting	Brine one day and then dry salted

brine-salted and the Romano cheese was brine-salted before being transferred to The Ohio State University. The dry salting of the Romano was completed at the University. The cheeses were stored for 1 year at a temperature of 50° ± 2° F. and a relative humidity of 75%.

The sampling techniques and methods for determining moisture and water-soluble nitrogen were the same as those used in previous work *(3, 4)*. Analyses for free amino acids and fatty acids were made at 1, 14, 30, 60, 90, 220, and 360 days of age. Analyses were made for free alanine, aspartic acid, glutamic acid, glycine, histidine, the combined leucines, methionine, threonine, and valine; and for free acetic, propionic, butyric, and a group of "higher" fatty acids. The free amino acids were measured by the paper chromatographic method, and the free fatty acids were measured by the direct chromatographic method previously described *(1, 3)*.

RESULTS

The results of analyses at various times of ripening are similar for Provolone and Romano cheese. For convenience the results for each cheese variety are presented separately.

Provolone cheese. The intensities of the characteristic flavor of the four Provolone cheeses during the ripening period are shown in Table 3. The cheese made with rennet extract exhibited the least flavor development, whereas the cheese made with the kid glandular preparation and the kid rennet paste showed the greatest and most rapid development of flavor.

TABLE 3

Development of characteristic flavor in Provolone cheese made with different enzyme preparations during 360 days of ripening at 50° F.

Sample No.	Source and type of enzyme[b]	Characteristic flavor score[a]					
		Age (days)					
		15	30	60	90	220	360
50	Rennet extract	0	0	0	0	0	0
51	Calf gland enzyme	0	0	0	0.5	2	2
52	Kid gland enzyme	0	¶	0.5	1.5	3	3.5
53	Kid rennet paste	0	¶	0.5	1	2	3.5

[a] Characteristic flavor: intensity from 0 to 4.
[b] Rennet extract also used with all glandular preparations.

The four different treatments of milk with coagulating and ripening enzymes produced practically the same development of propionic and acetic acids during the one year of curing. The propionic acid was detectable, but not measurable, at 1 day of age; it increased from approximately 0.10 mg. per gram of cheese solids at 14 days to 0.23 mg. at 370 days. Acetic acid in milligrams per gram of cheese solids at 1, 14, 30, 60, 90, 220, and 370 days of curing approximated 0.05, 0.15, 0.25, 0.32, 0.23, 0.32, and 0.40, respectively.

TABLE 4

Butyric acid present in Provolone cheese made with various enzyme preparations

Age at analysis	Butyric acid[a] in Provolone made with:			
	Rennet extract	Rennet plus calf glandular preparation	Rennet plus kid glandular preparation	Kid rennet paste
1	0.05	0.12	0.13	0.13
14	0.31	0.39	0.55	0.66
30	0.51	0.88	1.20	1.50
60	0.55	1.10	1.60	1.80
90	0.55	1.30	1.80	2.20
220	0.55	1.50	2.30	2.60
370	0.71	1.70	2.90	3.20

[a] Mg. acid per gram of cheese.

The free butyric content measured in the cheese at 1, 14, 30, 60, 90, 270, and 370 days is given in Table 4. As previously reported for cheese manufactured under varied conditions *(3)*, a definite relationship was found to exist between the kind of enzyme product used in the manufacture of the cheese and the development of free butyric acid in the cheese. Of the free fatty acids, only butyric could be related to the enzyme product. Even at 14 days this relationship was apparent, and at 60 days the concentration of the free butyric acid was directly related to the type of enzyme product, which in turn was related to the flavor

elopment of the cheese. The cheese made with rennet extract showed little rease in free butyric acid after the first 30 days, whereas the cheese made with yme preparation containing active lipase showed a gradual increase of free yric acid throughout the ripening period.

At 1 day of age free alanine, glutamic acid, leucines, and valine were de- table, but not present in measurable concentrations. As the cheese aged, the e leucines were present in the greatest concentrations, followed by the glu- nic acid, valine, and alanine. Glycine and threonine were not detected until er 60 days of storage, whereas methionine and histidine were present in some d absent from other samples at 60 days. The free amino acid values were not ntical in the four different samples, but the values could not be related to kind of enzyme product used in the manufacture.

The greatest changes in concentration occurred after 90 days of storage. A mparison of the free amino acid content at 90 and 370 days is given in Table 5. here was generally a marked increase in almost all amino acids between 90 and

TABLE 5

Concentrations of various free amino acids liberated at 90 and 370 days in the ripening of Provolone cheese made with various enzyme preparations

Treatment:	Rennet extract		Rennet plus calf glandular preparation		Rennet plus kid glandular preparation		Kid rennet paste	
Age in days:	90	370	90	370	90	370	90	370
Amino acids	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*
Alanine	1.10	2.20	1.80	2.60	1.30	1.90	1.40	2.30
Aspartic acid	0.80	1.80	1.10	2.20	1.10	1.60	+	2.40
Glycine	+[a]	1.30	0	1.10	+	1.40	+	1.10
Glutamic acid	1.60	5.80	1.90	6.10	2.00	5.60	2.10	6.30
Histidine	0	0.70	0	0	0	0.40	0	0.30
Leucine	3.40	5.90	3.70	7.20	3.80	6.80	3.20	7.60
Methionine	0.30	0.30	+	0	0.20	0.50	0.30	0.30
Threonine	0	0.20	0	0.20	0	0.10	0	0.20
Valine	1.50	2.10	0.70	1.70	1.30	2.00	1.40	2.30

[a] + indicates less than 0.1 mg/g cheese solids.

370 days. The free amino acids were present in relatively large quantities in the year-old cheese, with the leucines and glutamic acid being present in the highest concentrations.

One interesting observation was that as the cheese aged, the variation in the free amino acid content of the various cheeses became less. In the year-old cheese the amino acid contents were similar, with the exception of histidine and methio- nine. These acids were present in some cheeses and absent in others.

Romano cheese. The characteristic flavor scores of the four Romano cheeses are given in Table 6. As in Provolone, the cheeses made with rennet extract showed very little characteristic flavor development during the ripening period. The cheese made with kid enzyme products showed the greatest intensity of flavor development.

The degradation of fat was definite at 1 day, and all of the lower fatty acids were present in measurable amounts. As in Provolone cheese, the four different

TABLE 6

Characteristic flavor intensity of experimental Romano cheese during 370 days of ripening[a]

Sample No.	Source and type of enzyme	Characteristic flavor intensity					
		Age (days)					
		15	30	60	90	220	370
1	Rennet extract	0	0	0	0	1	1
2	Calf gland enzyme	0	0	0.5	0.5	3	3
3	Kid gland enzyme	0	0	0.5	1	3	4
4	Kid rennet paste	0	0.5	1	2	2.5	4

[a] Characteristic flavor; intensity from 0 to 4.

treatments of the milk with coagulating and ripening enzymes produced prac- tically the same development of propionic and acetic acids during 1 year of curing. Propionic acid in milligrams per gram of cheese solids at 1, 14, 30, 60, 90, 220, and 370 days of curing approximated 0.1, 0.12, 0.17, 0.23, 0.03, 0.36, and 0.40, respectively. Acetic acid at 1, 14, 30, 60, 90, 220, and 370 days of curing approximated 0.1, 0.3, 0.4, 0.52, 0.56, 0.58, and 0.63 mg. per gram of cheese solids, respectively.

TABLE 7

Butyric acid present in Romano cheese made with various enzyme preparations

Age at analysis	Butyric acid[a] in Romano made with			
	Rennet extract	Rennet plus calf glandular preparation	Rennet plus kid glandular preparation	Kid rennet paste
1	0.15	0.30	0.45	0.51
14	0.29	0.52	0.72	1.10
30	0.53	1.20	1.40	1.80
60	0.62	1.80	2.20	2.60
90	0.72	1.90	3.00	3.40
220	0.90	2.40	3.90	4.20
370	1.20	3.20	5.50	5.70

[a] Mg. acid per gram cheese solids.

The amount of free butyric acid in the cheese at 1, 14, 30, 60, 90, 220, and 370 days is shown in Table 7. The results are similar to those previously shown for Provolone cheese, except that the compounds are present in much higher con- centrations in Romano cheese. The relationship between lipase enzyme and char- acteristic flavor was observed and is apparent even in 1-day-old cheese.

Most of the free amino acids studied were present in the 1-day-old cheese, and only glycine and threonine were not detected. The greatest increase in free amino acid content occurred between 90 and 370 days of age.

The free amino and fatty acid content of the cheese at 90 and 370 days is shown in Table 8. The results are similar to those previously reported for Provo- lone, except that the compounds are present in much higher concentrations. Although there were differences in the amounts of various free amino acids, none of the variations could be related to the type of enzyme products used in the manufacture. The general pattern of free amino acid is the same as for Provo- lone.

TABLE 8

Concentrations of various free amino acids liberated at 90 and 370 days in the ripening of Romano cheese made with various enzyme preparations[a,b]

Treatment:	Rennet extract		Rennet plus calf glandular preparation		Rennet plus kid glandular preparation		Kid rennet paste	
Age in days:	90	370	90	370	90	370	90	370
Amino acids	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*	*(mg.)*
Alanine	2.00	3.00	2.50	2.80	1.90	2.60	2.20	2.40
Aspartic acid	2.40	3.80	2.30	3.00	1.90	3.40	2.90	3.00
Glycine	0	1.70	+	1.80	0	2.10	0	1.90
Glutamic acid	3.60	7.10	2.80	6.90	2.40	8.30	5.50	7.20
Histidine	0	0.70	0	0	0	0	0	+
Leucine	4.30	8.00	5.70	8.30	5.60	9.10	5.30	8.40
Methionine	+	0.40	+	0.60	+	0	+	0.10
Threonine	0	0.40	0	0.30	0	0	0	0.10
Valine	1.30	2.90	1.70	3.20	1.60	3.60	1.50	2.90

[a] Mg. acid per gram cheese solids.
[b] + indicates less than 0.1 mg. amino acid present per gram.

DISCUSSION

This study, conducted under carefully observed conditions, basically substantiates the earlier observations pertaining to the relationship of the enzyme product to the ripening of Italian cheese. Rennet extract, which contained no active lipase, did not result in characteristic flavor development. The liberation and concentration of butyric acid in this cheese is similar to that reported for Cheddar cheese *(6)*. The two kid preparations resulted in cheese with similar flavor and similar butyric acid contents, whereas the calf rennet paste produced more butyric acid than the rennet extract, but not as much as the kid product. The confirmation of the proposed relationship between enzyme product, flavor production, and butyric acid formation is considered good evidence for the importance of the enzyme product in the ripening of Italian cheese. The results also show that the hygienically unsatisfactory rennet paste is not essential, because the desired characteristic flavor developed and butyric acid was formed in the cheese made with purified enzyme preparations from glandular sources.

The lack of relationship noted between the free amino acid content of the cheese and the enzyme product is further evidence that a second and independent factor is involved in the ripening of both Romano and Provolone cheese. Although both the Romano and the Provolone were made from the same lot of raw milk, all of the Provolone samples were phosphatase-negative as a result of the high temperature received during the molding process. This would account for the lower rate of degradation of both fat and protein in the Provolone cheese. It might also account for the greater uniformity in the free amino acid content of Provolone cheese as compared to that of Romano cheese.

The concentration of many of the free amino acids showed tendencies to first increase, then decrease, and subsequently again increase in concentration as the ripening period progressed. Such changes in end-product concentration could not be related to the lipase enzyme preparations and, therefore, these changes probably reflect changes in the bacterial flora during ripening and in the utilization of certain amino acids in the metabolism of microorganisms. Thus, the compounds available at any given time would tend to direct the growth of different types of flora, depending on the enzyme system of the microorganisms. Therefore, to insure the accumulation of the desired end products and proper flavor productions, attention must also be given to those enzyme systems which enable the bacteria to utilize certain amino compounds as sources of carbon and energy. The differences in raw and pasteurized milk cheese may be explained not only by differences in active proteolysis but partially by the subsequent utilization of certain amino compounds by microorganisms or by chemical reaction with other compounds which provide the desired end result.

The presence of certain amino acids, such as glutamic acid and the leucines, in high concentrations in the 370-day-old cheese undoubtedly has an influence on the flavor characteristics of the cheese. However, the absence of any particular "free" amino acid in the cheese should not be considered as evidence that the compound is not liberated or is not important. Rather, this absence might only indicate the presence of an active enzyme system capable of converting the compound into a secondary compound, which in turn could be a part of the flavor. Therefore, attention might be given also to compounds that may be present in relatively low concentration during ripening, as related to their concentration in casein. Threonine, methionine, and histidine are notable examples of compounds present in low concentrations. These compounds have been shown to play an important part in bacterial metabolism, and their transformation may be important in the development of cheese flavor.

SUMMARY

Each of four vat lots of milk was treated with one of four different enzyme combinations and made into Provolone cheese. Another series of four vat lots of milk was treated in the same manner and made into Romano cheese. Various free amino and free fatty acids developed in the ripening cheese were measured chromatographically over a period of 1 year.

The rate of formation and the concentration of free butyric acid were found to be directly related to the type of enzyme product used in the manufacture of the cheese. The kid rennet paste and kid glandular lipase preparations resulted in cheese with the highest butyric acid content, the calf product was next, and the rennet extract resulted in a cheese with a very slow rate of butyric acid flavor production.

The enzyme product was not related to the accumulation of the free amino acids. A hypothesis of interconversion of amino compounds is discussed. The appearance of the desired cheese flavor was related to butyric acid and glutamic acid in Provolone cheese and to butyric acid in Romano cheese. Glutamic acid could not be related to the characteristic flavor of Romano. The leucines increased to a consistently higher level than did the glutamic acid in the ripening of Provolone cheese. Further work is in progress to investigate the significance of this finding.

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THE NEUTRAL CARBONYL COMPOUNDS IN BLUE-MOLD TYPE CHEESE

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The importance of methyl ketones as flavor constituents of cheese in which *Penicillium roqueforti* is the ripening agent was indicated by Starkle *(9)* and Hammer and Bryant *(2)* and more recently has been conclusively established by Patton *(6)*. The latter isolated pentanone-2, heptanone-2, and nonanone-2 by fractional distillation of the steam volatiles obtained from a rather large quantity of a domestic Blue cheese of good quality. Further investigation by this author indicates that heptanone-2 has a flavor and aroma typical of Blue cheese and that Blue cheese flavor could be simulated in salad oil by addition of this ketone and butyric acid *(7)*.

In light of the above reports the development of a simple and rapid test for qualitative and semi-quantitative evaluation of the ketonic flavor constituents of blue-mold cheese would seem desirable. Experience with a chromatographic procedure for the separation and identification of 2,4-dinitrophenylhydrazones of aldehydes and ketones *(5)* has led to the development of such a test.

EXPERIMENTAL PROCEDURE

Ten ½-lb. samples of blue-mold type cheese representative of the cheese sold at nearby retail outlets were obtained. No information concerning these samples other than the variety, brand, and source was available. The samples were examined for flavor, aroma, and mold growth.

Distillation of cheese samples. Fifty grams of cheese from which all traces of rind and outer portions had been removed was mixed in a Waring blendor with 50 ml. of water until homogeneous. The cheese suspension was poured into a 250-ml. Erlenmeyer flask containing several glass beads and one drop of Dow Corning Silicone Antifoam A. Thirty ml. of 0.2% 2,4-dinitrophenylhydrazine in 2 *N* HCl was added to a 125-ml. Erlenmeyer flask. The two flasks were connected to a Y-shaped distillation tube similar to that described by Van Slyke *et al. (11)* by means of tight-fitting rubber stoppers. The third arm of the distillation tube was fitted with a short piece of heavy-walled rubber tubing and a screw-clamp. The assembled apparatus was connected to a mechanical vacuum pump protected by a calcium sulfate moisture trap. The distilling apparatus was swirled rapidly and evacuated until a head of foam developed on the cheese mixture. The vacuum was interrupted by closing the screw-clamp, and the cheese mixture was agitated until the foam dissipated. This procedure was repeated until the cheese mixture boiled gently without appreciable foam formation when the screw-clamp was opened momentarily. The flask containing the 2,4-dinitrophenylhydrazine solution was then immersed in an ice-water bath,

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